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The long range goal of this laboratory is to identify integrin-associated signaling events that contribute to the constitutive migration of human breast cancer cells on the laminin extracellular matrix proteins. This project has shown that the heterotrimeric G-protein Gαi3 may link integrin activation and migration of MCF-10A cells via a cAMP signaling pathway. The focus of the remaining work on this project is to identify how each integrin subtype which binds laminin-1 contributes to the regulation of this and other pro-migratory signaling pathways.

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Introduction

In a screen of drugs that block the function of specific intracellular signaling molecules, it was found that an inhibitor of heterotrimeric G-protein mediated signaling blocked the integrin induced migration of breast epithelial cells on the extracellular matrix protein laminin-5. The work supported by this grant focused on understanding how integrin/laminin interactions regulate G-protein mediated signaling and ultimately, cell migration potential. My hypothesis states that, because integrins are capable of stimulating multiple signaling cascades, including G-proteins, and multiple integrin isotypes are expressed on any given cell, the migration potential is determined by the activation state of all expressed integrins at a point in time. Therefore, a strategy was developed to stimulate integrin subtypes individually through the production of integrin specific recombinant laminin domains, and analyze the activation state of heterotrimeric G proteins and other pro-migratory signaling cascades.The conclusions of this work to date demonstrate the importance of G-proteins in regulating integrin mediated cell migration, the successful production of recombinant laminin domains, and demonstration of cooperativity in integrin mediated signaling. In addition, the training goals of this grant were accomplished with the successful completion of the Ph.D. program and employment of the principle investigator.

Body

The first and objective of the project, to identify the heterotrimeric G protein Gαi3 as a component of the migration signaling pathway stimulated by engagement of β1 integrin to laminin-5, and was accomplished and the results published (Appendix1). We demonstrated that stimulation of the β1 integrin by the activating antibody TS2/16 both enhanced migration of non-migratory breast cells on laminin-5, and induced a concurrent rise in cAMP production. The enhanced migration could be mimicked by application of non-hydrolyzable cAMP analogs, and could be blocked by inhibitors of cAMP dependent protein kinase or the Gαi and Gαs classes of heterotrimeric G-proteins. Furthermore, we demonstrated that pertussis toxin, an inhibitor of the Gαi class, specifically ADP ribosylates Gαi3 in our model cell line (MCF-10A).

The second major objective, to identify the integrin subtypes used by MCF-10A cell to engage laminin-1, and to design, produce, and purify candidate domains of laminin-1 that are likely binding partners for those integrins was also accomplished (Appendix2). Six proteins derived from the EHS laminin-1 sequence corresponding to specific globular domains were produced in a baculovirus expression system. Based on silver-staining of protein elutions, each protein was purified to greater then 95% purity. Protein yields varied from 2-10μg/L. The molecular weight of each protein conforms closely to the predicted size following cleavage of carbohydrate chains. All six proteins are glycosylated via N- and not O-linkages based on gel shifts following cleavage by pan-specific N- and O- glycosidases.

In addition, I showed that intracellular calcium flux is required for adhesion to laminin-1, and that intracellular calcium flux is dependent upon the type of matrix and integrin engaged. Each matrix tested caused the release of a unique concentration of cytosolic calcium, and all were higher than cells plated on poly-L lysine. This is presumably due to the subtypes and quantities of integrins engaged. In order to demonstrate that simple clustering of integrin subtypes is also capable of stimulating

variable cytosolic Ca^{2+} flux, cells were plated in wells coated with antibodies directed against known laminin-1 binding integrins and intact laminin-1. Each of the integrins that influenced adhesion to laminin-1 stimulated an intracellular calcium release that was different from binding of the intact molecule. These results suggest that signaling events downstream of integrin activation synergistically determine the ultimate concentration of cytosolic calcium release upon matrix engagement. Integrin function is not the result of linear signaling cascades but a consequence of cooperation among cascades stimulated simultaneously by specific integrin subtypes. (Appendix2).

Unfortunately, none of the recombinant proteins functioned as efficient integrin ligands. This failure of the recombinant proteins to support adhesion may be due to altered conformations, low concentration, or artifacts of the baculovirus expression system. Because we believe the low yield of the system is mostly responsible for the lack of biological activity, these domains are currently being re-produced in a bacterial expression system.

Key Research Accomplishments:

1. Identification of the heterotrimeric G protein Gαi3 as a component of the migration signaling pathway stimulated by engagement of β1 integrin to laminin-5. Demonstration that cAMP modulation through Gα proteins is required and sufficient for enhanced migration of MCF-10A cells on laminin-5.

2. Identification of the integrins subtypes used by MCF-10A cell to engage laminin-1. The integrins α1β1, α3β1, and α6β1 contribute to adhesion of MCF-10A cells on laminin-1.

3. Demonstration that activation of integrin subtypes variably regulate intracellular calcium flux, a requirement for both adhesion and migration of epithelial cells.

4. Design, production, and purification of candidate domains of laminin-1 that are likely binding partners for α1β1, α3β1, and α6β1 integrins.

Reportable Outcomes:

1. Publication of a manuscript describing cAMP-dependant migration of breast cells on laminin-5 (Plopper G.E., Huff, J.L., Rust, W.L., Schwartz, M.A., Quaranta, V. (2000) *Molecular Cell Biology Research Communications* **4**, 129-135, Appendix A)

2. Publication of all portions of the dissertation that was generated during the course of this work. (Rust, W. L., Carper, S.W., Plopper, G.E. 2002. The promise of integrins as effective targets for anti-cancer agents. Revew. Journal of Biotechnology and Biomedicine. In Press.) (Appendix3).

3. Graduation with the degree of Ph.D., Biological Sciences, from the University of Nevada, Las Vegas. 12/01.

4. Employment as Post-Doctoral Researcher in the transplantation department of Navartis Pharma, AG, Basel, Switzerland.

Conclusions

The long range scientific goal of this work is to describe cellular mechanisms that contribute to the metastatic potential of breast cancer cells for the purpose of identifying targets for chemotherapy development. We show that heterotrimeric G-proteins are just one of multiple signaling cascades that regulate a key element of metastasis, cell migration. Furthermore, we demonstrate that the migration response is regulated in part through a cooperation between multiple activated integrins. Lastly, we built a model for analyzing the critical junctures in that cooperation using integrin-activating domains of the laminin-1 molecule. This model is currently being refined.

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Appenidces

Antibody induced activation of β1 integrin receptors stimulates cAMP-dependent migration of breast cells on laminin-5

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Key words: Extracellular matrix, metastasis, signal transduction

ABSTRACT

The β1 integrin-stimulating antibody TS2/16 induces cAMP-dependent migration of MCF-10A breast cells on the extracellular matrix protein laminin-5. TS2/16 stimulates a rise in intracellular cAMP within 20 minutes after plating. Pertussis toxin, which inhibits both antibody-induced migration and cAMP accumulation, targets the Gαi3 subunit of heterotrimeric G proteins in these cells, suggesting that Gαi3 may link integrin activation and migration via a cAMP signaling pathway.

INTRODUCTION

Laminins are a diverse group of heterotrimeric extracellular matrix proteins that constitute a major component of the basement membrane of epithelial tissues. The laminin-5 isoform, consisting of the α3, β3 and γ2 subunits, is abundantly expressed in the basement membrane of breast tissue [1] where it plays a role in mammary branching morphogenesis, and adhesion and migration of breast epithelial cells [2].

Evidence from both *in vitro* and *in vivo* studies support a functional role for laminin-5 in cell migration of both normal and malignant breast epithelial cells. Our laboratory has previously shown that *in vitro*, laminin-5 is the preferred adhesive substrate for breast epithelial cells [1]. In haptotactic migration assays, non-tumorigenic breast cell lines fail to migrate significantly on laminin-5, whereas laminin-5 supports migration of highly malignant breast cell lines. *In vivo*, laminin-5 expression is enhanced in invading regions of metastatic breast tumors[3]. In addition, an altered conformation of laminin-5, resulting from proteolytic cleavage of the γ2 chain by matrix metalloprotease 2, is found at sites of tissue invasion, and this cleavage stimulates migration of otherwise non-migratory breast cells *in vitro* [4]. How laminin-5 contributes to progression of tumorigenic cells from the stationary to malignant phenotype is unknown, but may involve stimulating enhanced migration of breast cells.

Cells interact with laminins primarily through integrin receptors [5]. Ligand induced signal transduction by integrin/laminin binding regulates intracellular pH, tyrosine phosphorylation, inositol lipid metabolism, and calcium (Ca^{2+}) oscillations [6]. Signaling molecules known to associate with integrins receptors include protein tyrosine kinases, serine/threonine kinases, phospholipid kinases and lipases, ion channels, and members of the rho family of small molecular weight GTP binding proteins [6]. Laminin-5 is recognized by the α3β1, α6β1 and α6β4 integrin receptors in a number of cell types, and the functional consequence of these interactions depend on the integrin receptor engaged. For example, ligation of laminin-5 with the α6β4 integrin receptor supports branching morphogenesis and hemidesmosome formation in breast epithelial cells [2], while interaction with α3β1 integrin supports migration of these same cells *in vitro* [7]. Little information is currently available on the specific signaling pathways triggered during these events.

While investigating the role of the α3β1 integrin in motility of breast epithelial cells, we observed that haptotactic migration of the immortalized breast epithelial cell line MCF-10A on laminin-5 was stimulated by direct activation of the β1 integrin receptor with the β1-activating monoclonal antibody TS2/16. Migration was dependent on intracellular cAMP signaling, and TS2/16-promoted a rise in intracellular cAMP levels that occurred 20 minutes after plating on laminin-5. Migration and cAMP accumulation were inhibited by treatment of the cells with pertussis toxin, a compound that inactivates the α subunit of the inhibitory class of heterotrimeric G proteins via ADP-ribosylation. We show that the Gαi3 isoform is a target for ribosylation by pertussis toxin in these cells. Together these data present evidence that the β1 integrin participates in the regulation of MCF-10A cell migration on laminin-5 through a cAMP-signaling pathway involving Gαi3. This is the first description linking integrin activation to signaling through heterotrimeric G proteins.

MATERIALS AND METHODS

Cells. MCF-10A cells were maintained in DFCI medium according to Band and Sager [8]. MDA-MB-231 cells were cultured as described [1]. Rat 804G cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 1X Glutamine Pen-Strep solution (Irving Scientific). 804G cell conditioned medium was collected after 3 days of culturing and was clarified by centrifugation at 1500 x g.

Reagents. Mouse monoclonal antibodies against human integrin α3 (clone PlB5) and β1 (Clone P4ClO) were purchased from Gibco (Gaithersburg, MD). Mouse monoclonal antibody clone P5D2 against human β1 integrin was purchased from Chemicon (Temecula, CA). Purified rat anti-mouse β1 antibody 9EG7 was purchased from Pharmingen (San Diego, CA), and dialysed against PBS to remove sodium azide. Mouse monoclonal anti-human, activating β1 integrin antibody TS2/16 (in ascites form) was generously provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA). Anti-rat laminin-5 monoclonal antibody TRl was produced in-this laboratory [9]. Both TS2/16 and TR1 were purified with a protein G affinity chromatography kit (Pierce, Rockland IL). SQ22536 was purchased from Biomol (Plymouth Meeting, PA) and pertussis toxin from List Biological Laboratories, Inc. (Campbell , CA). 8-Bromo-cAMP, dibutryl cAMP, H-89, and forskolin were purchased from Calbiochem (San Diego, CA).

Adhesion and migration assays. Adhesion and migration assays were performed as previously described [1]. For anti-integrin antibody blocking experiments, antibodies were incubated with cells for 30 minutes before adding to assay wells, and were present throughout the assays.

cAMP determination. Cells were collected by brief trypsinization, blocked with trypsin inhibitor, washed in DMEM, counted, resuspended at 1 x 10^6 cells/ml, and incubated at 37°C in migration medium/1 mM isobutylmethylxanthine (Sigma) to block phosphodiesterase activity. After 30 minutes, anti-integrin antibodies (TS2/16 or P5D2) were added, and cells were incubated at 37°C for an additional hour. Control cells were suspended in DMEM/1 mM isobutylmethylxanthine alone during this time. Cells (1 x 10^6/plate) were then plated on 35 mm dishes coated with affinity-captured laminin-5 [1] and incubated at 37°C for the indicated times. Cells representing the 0 time point were immediately retrieved from the dishes, collected by centrifugation, and lysed in cold cAMP extraction solution (95% ethanol, 5% 0.1 N HCI). After 10, 20, 30, and 90 minutes non-adherent cells were aspirated, plates were washed with PBS, and cAMP extraction buffer was added to the adherent cells. The PBS washes from each plate were centrifuged to collect loosely adherent cells, and these were added back to the appropriate extraction. All samples were kept on ice in cAMP extraction buffer for 2 hours, then centrifuged to pellet precipitated protein. Protein was dissolved in 0.1 N NaOH and concentrations were determined with the BCA microassay (Pierce). Supernantants were evaporated and cAMP measured using a cAMP EIA kit (Perseptive Diagnostics, Inc., Cambridge MA) as directed by the manufacturer. cAMP amounts were normalized to total protein in each sample and expressed as fmol/μg protein.

ADP ribosylation assay.

Membrane Preparation:

Membranes were isolated from MCF-10A cells by lysis in ice-cold 10 mM HEPES pH 7.5, 3 mM $MgCl_2$, 2 mM EDTA containing 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml Pefabloc SC (Boehringer Mannheim Biochemicals, Indianpolis, IN). Cells were scraped, centrifuged to pellet nuclei, and the supernatant was collected. Membranes were pelleted from supernatant by centrifugation at 13,000 x g for 30 minutes at 4°C; and the pellets were resuspended in lysis buffer. Protein concentrations were determined by BCA protein assay (Pierce).

ADP-ribosylation and Immunoprecipitation:

ADP-ribosylation reactions were performed as described [10]. Final reaction conditions were as follows: 100μg membrane protein was suspended in 20 mM thymidine, 1 mM ATP, 1 mM GTP, 1 mM EDTA, 20 mM HEPES, pH 7.5 with or without 7.5 μg pertussis toxin (activated prior to experiment by incubation for 10 minutes at 37°C in 20 mM DTT, 20 mM HEPES, pH 7.5) and 25 μCi ^{32}P-NAD (Specific activity = 30 Ci/mM, New England Nuclear catalog # BLU023). Reactions proceeded for 45 minutes at 30°C and were stopped by chilling to 4°C followed by a wash with 20 mM HEPES pH 7.5, 1 mM EDTA and 1 mM DTT. For SDS-PAGE analysis, membranes were solubilized in 50 μl Laemmli sample buffer (LSB), heated for 5 minutes at 100°C and separated on a 12% SDS-polyacrylamide gel. ^{32}P-labeled proteins were detected by autoradiography of dried gels using Kodak X-Omat AR film with intensifying screens. For immunoprecipitations, ribosylated membrane proteins were solubilized in RIPA buffer containing protease inhibitors and were incubated with the following G-protein α subunit-specific peptide antibodies: I-20, specific for Gαi -1; C-10, specific for Gαi-3 (Santa Cruz Biotechnology). Immune complexes were captured by incubation with A/G agarose (Santa Cruz Biotechnology), solubilized by boiling in LSB and analyzed by SDS-PAGE as described.

RESULTS AND DISCUSSION

The β1 integrin activating antibody TS2/16 stimulated MCF-10A migration on laminin-5.

The non-tumorigenic breast cell MCF-10A remains statically adherent to laminin-5 via the α3β1 integrin [1]. In haptotactic Transwell filter migration assays, these cells demonstrated only modest migration towards laminin-5. When pre-incubated with TS2/16, however, MCF-10A cells increased their migration in a dose-dependent manner towards laminin-5 (Fig 1). TS2/16-treated cells also exhibited increased adhesion to laminin-5 (Fig. 2). These effects are not observed with other β1 targeting antibodies (P5D2, 9EG7, Fig. 1; 9EG7, Fig. 2) or with TS2/16 on other substrates (data not shown). TS2/16 therefore stimulated a signaling pathway that, concurrent with laminin-5 binding, led to enhanced cell migration. This pathway is dependent upon binding of the α3β1 integrin, as pretreatment of the cells with the α3 integrin-blocking antibody P1B5 completely blocked TS2/16-stimulated migration on laminin-5 (Fig 1).

The strength of cell adhesion to extracellular matrix ligands varies over a wide range, and is under the control of both intracellular and extracellular cues. Work by Lauffenburger [11] suggests that very tight or very loose cell adhesion to matrix proteins will not support cell migration, and that migration occurs only when a medium-strength of adhesion is achieved. Thus, varying the potency of adhesion of integrin receptors for their ligands may be a critical step for regulating cell migration. It is possible that TS2/16 stimulated migration in these cells by changing the strength of adhesion between α3β1 integrin and laminin-5, either directly or via activation of internal signaling pathways.

Alternatively, it is plausible that TS2/16 induced a conformational change in the β1 integrin that mimicked binding to a pro-migratory form of laminin-5, such as those created through proteolytic processing. For example, cleavage of the γ_2 subunit of laminin-5 creates a conformation on which MCF-10A cells migrate constitutively [4, 12]. A pro-migratory laminin-5 can be converted to one that inhibits cell migration through cleavage of the α3 chain [13]. In both instances it is assumed that proteolytic processing masks or unmasks a pro-migratory domain on the intact laminin-5 trimer. This theory is also supported by studies showing that integrin activation by TS2/16 will rescue the growth of MCF-10A cells inhibited by treatment with laminin-5 blocking antibodies [14].

MCF-10A cell migration on laminin-5 is modulated by cAMP.

To define the mechanisms by which TS2/16 stimulated MCF-10A cell migration on laminin-5, we added inhibitors of known signaling molecules to antibody-stimulated cells in haptotaxis migration assays. We found that SQ22536, an inhibitor of adenylate cyclase, and H-89, an inhibitor of cAMP dependent protein kinase, completely blocked TS2/16 stimulated migration on laminin-5 (Fig. 3). In addition, pharmacological enhancement of cAMP levels with either forskolin or the non-hydrolyzable cAMP analogs 8-bromo-cAMP and dibutyryl cAMP were sufficient to enhance migration of MCF-10A cells on laminin-5 to levels stimulated by TS2/16 (Fig. 3). These data established that cAMP was required for enhanced migration of MFC-10A cells on laminin-5.

Because adenylate cyclase activity is governed by different classes of heterotrimeric G proteins we exposed MCF-10A cells to pertussis toxin (an inhibitor of the Gαi class) and cholera toxin (an inhibitor of the Gαs class). While both pertussis and cholera toxin partially blocked serum stimulated migration of MCF-10A cells, only pertussis toxin blocked TS2/16 stimulated migration on laminin-5 (Fig. 4). These data demonstrated that the specific pathway triggered by TS2/16 and laminin-5 was susceptible to regulation by Gαi rather than Gαs proteins. This is consistent with our observation that numerous chemokines that modulate cAMP through Gαs (bombesin, bradykinin, adrenaline) raised cAMP levels but failed to stimulate migration in our cells (G.E. Plopper, unpublished data).

Chemotactic migration of many cell types is inhibitable by cholera and pertussis toxins [15, 16]. While pertussis toxin allows for unchecked cAMP production in the short term, prolonged pertussis toxin exposure suppressed cAMP levels in our cells, likely because of long-term desensitization of this pathway [17]. Although O'Conner et al. [18] reported that α6β4 expression suppressed cAMP levels in migrating breast cancer cells, no evidence has been published linking cholera and pertussis-sensitive signaling pathways to integrin-activated signaling.

TS2/16 stimulated a rise in intracellular cAMP via a pertussis toxin-sensitive signaling pathway.

Since pertussis toxin alters intracellular cAMP levels, and cAMP modulation was sufficient to enhance migration in our cells, we examined the levels of cAMP in TS2/16-stimulated cells plated on laminin-5. Within 20 minutes after plating, cAMP levels were raised approximately four-fold in TS2/16 treated cells. This peak occurred within the time frame of integrin signaling [6]. Enhanced cAMP accumulation was specific to TS2/16, and not a product of integrin clustering, as neither cells treated with the non-activating β1 antibody P5D2 nor cells plated on laminin-5 without antibodies exhibited enhanced cAMP production (Figure 5A). Pre-incubation with pertussis toxin completely eliminated this peak but did not significantly affect basal cAMP levels (Figure 5B). Concurrent stimulation by laminin-5 adhesion and TS2/16 are required, as cAMP levels did not change in suspended cells treated with TS2/16 (G.E. Plopper, unpublished). It appeared, therefore, that the combination of intact laminin-5 and TS2/16 pretreatment stimulated a signaling pathway involving cAMP that was specifically blocked by pertussis toxin.

Pertussis toxin ADP-ribosylated Gαi3 in MCF-10A cells.

Pertussis toxin ADP ribosylates the Gαi class of heterotrimeric G proteins. To determine the repertoire of Gαi subunits expressed in MCF-10As we performed Western blot analysis of whole cell lysates and isolated membrane fractions using polyclonal antibodies raised against specific G protein subunits. These studies revealed that MCF-10A cells expressed Gαi1 and Gαi3, but not Gαi2 (data not shown). To establish the targets of pertussis toxin in these cells we carried out ADP-ribosylation assays in the presence of ^{32}P-NAD. Addition of pertussis toxin specifically induced the ribosylation of a 43 kDa protein (Fig. 6, lane 2). No ^{32}P labeled proteins are detectable without addition of pertussis toxin (Fig. 6, lane 1). The molecular weight of the ribosylated protein was consistent with that of the α subunits of heterotrimeric G proteins. The identity of this protein was determined by immunoprecipitation of the ribosylated membrane proteins with Gαi1 and Gαi3

antibodies. Antibody C10, which reacts primarily with Gαi3, immunoprecipitated a band of 43 kDa (Fig. 6, lane 4). The anti-Gαi1 antibody I-20 failed to precipitate any ADP ribosylated proteins in MCF-10A cells (Fig. 6, lane 3), but did precipitate a 43 kDa band from a control cell line, MDA-MB-231 (Fig 6., lane 5). Therefore, pertussis toxin ribosylated Gαi3, but not Gαi1 in MCF-10A cells.

In addition to controlling adenylate cyclase activity, Gαi3 is associated with and activates amiloride-sensitive Na^+ channels [19], which are expressed in many epithelial cells including breast. These channels are also regulated by the actin cytoskeleton [20] and cAMP dependent protein kinase [21], suggesting that Gαi3 may link integrin-mediated actin polymerization, cAMP signaling, cAMP dependent protein kinase activity, and amiloride-sensitive channel activation. Curiously, amiloride also suppresses lung metastases from breast tumors [22]; our data suggest that it may do so, at least in part, by inhibiting tumor cell migration.

In conclusion, we report that the β1 integrin-stimulating antibody TS2/16 induced migration of MCF-10A cells on laminin-5 that was dependent upon cAMP linked signaling. TS2/16 also stimulated a rise in intracellular cyclic AMP within 20 minutes after plating on laminin-5. Both the enhanced migration and cAMP peak were inhibited by pertussis toxin. Pertussis toxin targeted the Gαi3 subunit of heterotrimeric G proteins in these cells. This evidence suggests that the β1 integrin participates in the control of MCF-10A cell migration on laminin-5 via a cAMP signal pathway regulated by Gαi3. This form of signaling, beginning with an external stimulus of the integrin receptor, is referred to as "outside-in signaling" to differentiate it from changes in integrin function resulting from activation of internal signaling pathways [6]. We propose that TS2/16 mimics the effects of proteolytic processing of laminin-5 by forcing the α3β1 integrin into a conformation formed by binding pro-migratory forms of laminin-5. We are currently examining the effect of these proteolytic modifications on intracellular signaling activities in MCF-10A cells. Because acquisition of a migratory phenotype is required for malignant progression of tumorigenic breast cells, elucidating pathways involved in enhanced migration of breast may lead to discovery of novel targets for anticancer therapies.

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Figure Legends

FIG. 1. The integrin activating antibody TS2/16 stimulates migration of MCF-10A breast cells on laminin-5. Indicated concentrations of TS2/16 were added to MCF-10A cells in a minimal medium lacking serum or other growth factors (MM) 15 minutes prior to plating in laminin-5 migration assays, and migrated cells were counted 18 hours later. As controls, cells were plated in the presence of 10% serum, irrelevant mouse ascites (FM3 ascites), antibody purified from irrelevant ascites (FM3 pure), or non-fat dried milk (blotto). Results are expressed as the mean of eight measurements on two filters using 300 X magnification, +/- standard deviation.

FIG. 2. TS2/16 antibody increases adhesion of MCF-10A cells to laminin-5. Cells were incubated in MM with 50 μg/ml of TS2/16 or 9EG7 antibodies for 15 minutes, then were plated on affinity-captured laminin-5 for 30 minutes and adhesion quantified by measuring absorbance of crystal violet-dyed cells at 595 nm. Affinity capture was accomplished by successive addition of indicated concentrations of TR1 antibody, blotto, and 804G-conditioned medium containing soluble laminin-5. As a control, cells were incubated with no antibodies prior to plating. Results expressed as statistical mean +/- standard deviation (n=8).

FIG. 3. Enhanced cAMP levels induce migration of MCF-10A cells on laminin-5. Cells were incubated in MM supplemented with 50 μg/ml TS2/16, 50 μg/ml P1B5, 250 mM SQ22536 (SQ), 4 μM H89, 5 nM forskolin (FSK), 500 μM dibutyryl cAMP (db cAMP), or 500 μM 8-bromo-cAMP (8-Br-cAMP) for 15 minutes prior to adding to laminin-5 migration assays. As a control, cells were plated in the presence of serum or in MM on filters lacking laminins (blotto). Results are expressed as in Figure 1.

FIG. 4. Pertussis toxin inhibits TS2/16-stimulated migration on laminin-5. MCF-10A cells were suspended for 30 minutes in MM supplemented with either 10% serum or 50 μg/ml TS2/16. 100 ng/ml pertussi toxin (PT), 100 ng/ml cholera toxin (PT), were added 15 minutes prior to plating cells in laminin-5 migration assays. As a control, cells suspended in MM were added to filters coated with blotto alone. Results expressed as in Figure 1.

FIG. 5. Pertussis toxin inhibits a cAMP peak in TS2/16-stimulated cells. MCF-10A cells were suspended in MM supplemented with 20 μg/ml TS2/16 or P5D2 antibodies and plated on laminin-5 for the indicated time, then lysed and assayed for total cAMP content by ELISA assay. As a control, cells were plated in the absence of antibodies (CTL). The experiments were performed (A) in the absence (-PT) or (B) presence (+PT) of 100 ng/ml pertussis toxin. Results are normalized to total cell protein for each time point and represent the mean of triplicate measurements for four experiments, +/- the standard error of the means.

FIG. 6. Pertussis toxin specifically ADP ribosylates Gαi3 in MCF-10A cells. 100 μg of cell membranes were incubated with 25 μCi ^{32}P-NAD in the presence (lanes 2-4) or absence (lane 1) of 7.5 μg activated pertussis toxin. Pertussis toxin-treated lysates were immunoprecipitated with anti-Gαi1 (I-20, lane 3) or anti-Gαi3 (C10, lane 4) antibodies. As a control, Gαi1 was immunoprecipitated from ^{32}P-NAD labeled lysates of pertussis toxin-treated MDA-MB-231 cells (CTL, lane 5). Migration of molecular weight standards is shown at left.

Appendix 2

LAMININ-1 DOMAINS AS SPECIFIC ACTIVATORS OF INTEGRIN SUBTYPES

Abstract

Cells interact with laminin-1 with as many as six different integrin heterodimers. Here I have identified integrins □6□1, □1□1 and potentially □3□1 as laminin-1 receptors used by MCF-10A cells. I hypothesize that MCF-10A cell response to laminin-1 engagement is a product of the cumulative effect of engagement of all three integrins, stimulating at least two separate intracellular signaling cascades. To test this hypothesis, I identified intracellular calcium flux as an downstream marker of integrin signaling response, and demonstrated that clustering of independent integrins and different integrin ligands stimulate unique intracellular calcium flux. In order to gauge the impact of each integrin on calcium flux, I constructed six fragments of laminin-1 in a baculovirus system that likely contained integrin binding sites. Each of these were screened for the ability to act as adhesive substrates for MCF-10A cells. My results indicate that recombinant domains of laminin produced by this method are not suitable integrin ligands.

Introduction

It is tempting as a researcher to fit biological molecules into linear cascades of specific function. It is becoming more and more evident, however, that most molecules, especially those involved in signaling, are components of a balancing act between intracellular events whose ultimate outcome can only be predicted by observing the net influence of intracellular conditions within a specific time frame. For example, Ras is recognized as a molecule that promotes cell growth via MAP kinase stimulation. In the absence of PI-3 kinase, or NF-kB activation, Ras stimulation results in the opposite effect, apoptosis [1]. It can therefore be postulated that specific function can not be attached to particular molecules without taking into account other cellular conditions. In order to gain a true understanding of integrin function, the same paradigm must be applied.

To date, a great number of signaling cascades (kinase activation, phospholipid metabolism, small and large G-proteins, ion current, intracellular pH) and cellular functions (proliferation, death, migration, embryogenesis, gene transcription and protein secretion) have been attributed to integrin activation. Since a cell will engage it's matrix ligand with only a few integrin subtypes, it is impossible to rationalize the specificity of cellular outcomes based on linear, single-function signal cascades. The hypothesis proposed here is that the consequence of integrin ligation is an emergent property dependent upon the activation state of all expressed integrins. The specificity of integrin function is a result of cooperative balance between integrin stimulated signal cascades.

For this hypothesis to be reasonable, integrins with common substrate specificity must be capable of stimulating different signal cascades, and must be able to communicate by signal cascade overlap. As an example of similar integrins sending different signals, integrins bound to collagen type IV can either stimulate or inhibit RhoA activity depending on the specific integrins engaged, and time course of engagement [2]. There are several examples in support of the integrin communication by signal cascade overlap. Certain epithelial cells adhere strongly to laminin-5 via

□6□1, and weakly to laminin-1, where both □3□1 and □6□1 are engaged. Microinjection of the □3 cytodomain disrupts □6□1 mediated adhesion to laminin-1, indicating that □3□1 transdominantly regulates □6□1 activity [3]. This communication by integrin receptors is also demonstrated in the regulation of phagocytosis and migration by macrophages [4]. In this case, □v□3 blocks □5□1 function through inhibition of CaMKII. In myocardial muscle cells, transfection with integrin □7 stimulates cell adhesion to laminin-1, but inhibits the ability of native □5 to bind to fibronectin [5].

In order to understand how these emergent properties of integrins are achieved, it is necessary to be able to stimulate individual integrin subtypes alone and in combination, across a variety of cell types. For this I chose to produce recombinant fragments of the likely integrin binding domains of laminin-1, and screened those for the ability to bind one, and only one integrin subtype. Laminin-1 was chosen because it is recognized by six integrin subtypes (□1□1, □2□1, □3□1, □6□1, □7□1, □6□4) and is the most well researched of all the laminin isoforms.

In addition to integrin specific adhesion molecules, it was necessary to identify a marker of dynamic integrin signaling that was likely the result of cooperation between integrin signaling cascades. Intracellular calcium (Ca^{2+}) flux is a likely candidate as it is a consequence of, and required for, integrin mediated adhesion and migration [6 and Figures. 1-3]. Increases in Ca^{2+} are seen upon cell attachment to ECM in platelets, macrophages, neutrophils, osteoclasts, smooth muscle, epithelial, and embryonic stem cells [7, 8, 9]. The complex spatio-temporal aspects of Ca^{2+} signaling make it well suited for the modulation of highly coordinated processes such as migration, which require an asymmetric regulation of cell adhesion, with formation/strengthening at the front and disassembly/weakening at the rear [10]. Aside from being a factor which regulates integrin affinity and avidity, several proteins which modulate F-actin assembly or maintenance of focal adhesions are also regulated by calcium flux. These include calreticulin, calcineurin, calmodulin, calcium and integrin binding protein, gelsolin, calpain, and calcium/calmodulin dependent kinase II (CamKII) [4, 11, 12, 13]. The variety of mechanisms by which Ca^{2+} can be regulated also indicate that this is a good molecule for analyzing dynamic regulation by the family of integrin receptors.

The mechanism by which calcium fluxes are propagated and maintained by adherent and/or actively migrating cells is not completely understood. It is generally accepted, however, that intracellular calcium flux results from calcium entry form both internal and external sources. Nearly all cells carry receptor-operated and voltage-operated channels in the plasma and ER membranes, and store operated Ca^{2+} channels which are activated by a decrease of Ca^{2+} in the ER [14].Opening of Ca^{2+} channels of intracellular Ca^{2+} stores occurs via inositol-1,4,5-trisphophate (IP_3) binding, which is produced by the action of membrane bound phospholipase C (PLC) from the substrate inositol-4,5-bisphophate (PIP_2). Sustained Ca^{2+} release is thought to be achieved by the opening of Ca^{2+} release activated channels (CRAC) of the plasma membrane [12]. The signaling molecules PLC□1, CaMKII, calcineurin, and calreticulin are all implicated in stimulating Ca^{2+} influx through plasma membrane channels [8, 11].

Although it has been reported that integrin-ECM interactions lead to IP_3 production, no direct link between integrins and PLC activation has yet been established. A convincing model of indirect activation, however, has recently emerged. In this model, phosphatidylinositol-3,4,5-trisphophate (PIP_3) interacts with PLC□, thus targeting PLC□ to the membrane and contributing to its activation. PIP_3

is produced from PIP_2 by the action of phosphatidylinositol-3 kinase (PI-3K), an integrin associated kinase. Integrin binding activates PI-3K through the activation of FAK [12]. Published data in support of this model include demonstration that PI-3K regulates PLC mediated Ca^{2+} signaling [15], PI3 kinase targets PLC to the membrane [16], integrin binding activates PI3 kinase, and PI3 kinase promotes and is required for breast carcinoma invasion [17].

In this research, I identify 3 integrins expressed by the breast epithelial cells MCF-10A and MDA-MB-231 as potential receptors for intact laminin-1. I show that intracellular calcium flux is required for adhesion to laminin-1, and that intracellular calcium flux is dependent upon the type of matrix and integrin engaged. Furthermore, I describe the production of six recombinant fragments of laminin-1 by baculovirus expression system. Unfortunately, none of the recombinant proteins functioned as efficient integrin ligands, and the potential causes of this result are discussed.

Materials and Methods

Calcium Detection

Cells were serum starved for 45 minutes and trypsinized until all cells had released the substrate (~10 minutes). Trypsin was neutralized by 5mg/ml trypsin inhibitor (Boehringer Mannheim, Germany) in DME. Cells were then washed in DME and held in suspension for 30 minutes at room temperature in DME supplemented with 50mM HEPES pH 7.4 and 5□M Fluo-3 AM (Molecular Probes, Eugene, OR). 120,000 cells were plated per well in a 96 well plate coated as in adhesion assays with poly-L lysine, matrix proteins, or anti-integrin antibodies. Fluo-3 fluorescence was measured over time at 495nm excitation and 535nm emission in an automated fluorescent plate reader (Tecan SPECTRAFluor, Research Triangle Park, NC) warmed to 37°C. Background auto fluorescence of coated wells containing media only was subtracted from emission fluorescence at each time point.

Virus construction

Domains of murine laminin-1 were amplified by polymerase chain reaction (PCR) using sequence specific primers designed to generate single products with endonuclease specific 5' and 3' ends (Table 1). Laminin-1 cDNAs were generously provided by Y. Yamada [18]. The endonuclease digested PCR products were subcloned into the pBACgus-6 transfer plasmid (Novagen, Madison WI) in frame with the gp64 promoter, secretion signal sequence, and either a 5'or 3' 6X Histidine repeat. The cloned product was dideoxy sequenced in both directions to confirm proper insertion of the cDNA into the multiple cloning site of the plasmid. Recombinant baculovirus was generated by cotransfection of the pBACgus-6/domain transfer plasmids with linearized baculovirus (BacVector-3000 triple cut virus DNA, Novagen) into *Spodoptera frugiperda* 9 (*Sf*9) cells maintained in Sf-900 II SFM media (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gemini, CA), 100 U/ml penicillin G sodium, and 100 □g/ml streptomycin sulfate (Life Technologies). Successfully recombinant transfectants were identified by 5-Bromo-4-Chloro-3-indolyl-beta-D-Glucuronic acid (Bio-World, Dublin, OH) digestion and purified by three rounds of plaque purification. Cultured supernates were analyzed for domain expression by immunoblot analysis with anti-penta his monoclonal antibodies (Qaigen, Valencia, CA). Transfected cell supernates were subsequently used to generate high-titer stocks of recombinant viruses for future infections.

Protein production and purification

Sf9 cells were infected at >10pfu/cell and incubated at 29°C for 1-2 days in Sf-900 II SFM media supplemented with 100 U/ml penicillin G sodium, and 100 □g/ml streptomycin sulfate. Cells were collected and lysed in insect cell lysis buffer (10mM Tris-Cl pH 7.5, 130 mM NaCl, 1% Triton X-100, 10mM sodium phosphate pH 7.5, 10mM sodium pyrophosphate) supplemented with a 1:40 dilution of protease inhibitor cocktail for use in poly (Histidine) tagged proteins (Sigma-Aldrich, St. Louis, MO) for 45min at 4°C and centrifuged at 10,000G for 45min at 4°C. Ni-NTA agarose beads (Qiagen) were incubated in both cell lysate and cell supernate supplemented with 1mM Phenylmethylsulfonyl fluoride for four hours at 4°C and collected. Beads were pooled and sequentially washed with forty volumes wash buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl) supplemented with 80mM imidazole, and sixty volumes wash buffer supplemented with 5mM imidazole. Protein fractions were collected in elution buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl, 250 mM imidazole) and tested for purity by silver staining (Silver Stain Plus, Bio-Rad Laboratories, Hercules, CA). Each domain used in these studies was >95% pure. Typical yields ranged between 20-100 □g/L.

Adhesion Assays

Wells of a 96 well plate were coated for 1 hour at room temperature with laminin-1 or recombinant proteins at the indicated concentrations in elution buffer (see above). Negative control wells were coated for the same time in blotto (phosphate buffered saline (PBS) containing 5% nonfat dry milk in (pH 7.4) and 0.1%Tween 20). Uncoated surfaces were blocked by incubation at room temperature for 1 hour in blotto, and thoroughly washed in PBS. Cells were trypsinized and resuspended in adhesion media (DME, 0.25% heat-inactivated bovine serum albumin (BSA), 50 mM HEPES pH 7.4) at a concentration of 2.4 $*10^6$ cells/ml. Cells were kept in suspension at room temperature for 30mins with the indicated treatments. 120K cells were plated per well and incubated in a tissue culture incubator at 37°C for 30 mins. Non-adherent cells were removed by gently rocking the plates upside down in PBS for 15 minutes. Adherent cells were fixed for 15 minutes in 3.7% formaldehyde and stained for 15 minutes in 40% methanol/0.5% crystal violet. Adherent cells were quantified by manual counting using a 20X objective or by measuring absorption at 595nm following cell lysis in 1%SDS.

Glycosylation Analysis

To determine the extent of glycosylation, approximately 2□g of each domain protein was cleaved with peptide: N-Glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) according to manufacturers protocol and O-Glycosidase (Roche, Indianapolis, IN). For O-Glycosidase cleavage, proteins were denatured for 10 mins at 100°C in 0.1M □-Mercaptoethanol and 0.1% SDS. After cooling, samples were cleaved with 0.5 mU enzyme in 10% NP-40, 0.1M phosphate buffer pH 6, and 500□g/ml bovine serum albumin. All glycosidase reactions were allowed to proceed for 8 hours at 37°C.

Immunofluorescence Microscopy

Coverslips were coated overnight at 4°C with laminin-1 or domain proteins in elution buffer (see above). MDA-MB-231 cells were plated at a density of $6X10^5$ cells/ml in DME supplemented with 50mM HEPES pH 7.4 and 0.25% BSA and allowed to adhere for 90 minutes at 37°C. Adherent cells were fixed with 3%

paraformaldehyde and permeabilized in 0.2% Triton X-100 for 15 minutes each. Non specific antibody binding was blocked by 1 hour incubation with 3% BSA in PBS. Focal adhesions were visualized by staining for one hour with 1:400 dilution of anti-vinculin monoclonal antibody (Sigma Aldritch) in blocking solution followed by 1 hour incubation with 1:2000 dilution of rhodamine-conjugated anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR). F-actin was stained with 1:40 dilution of FITC-conjugated phalloidin (Molecular Probes) following incubation with secondary antibody. Stained cells were visualized with a Zeiss LSM Confocal microscope using a 40X objective.

Statistics

Statistical significance was determined by the ANOVA/T-test with a 95% confidence interval using JMP statistical software (Altura Software, Pacific Grove, CA).

Results

Integrin engagement stimulates varied intracellular calcium release.

Cells engage ECM matrices with only a subset of expressed integrins, depending on matrix specificity. Although there is some overlap, different subsets of integrins are engaged by each matrix. In order to determine if different matrices stimulate matrix-specific intracellular Ca^{2+} release, identical numbers of MCF-10A cells were pre-incubated with a fluorescent cytosolic Ca^{2+} dye (Fluo-3 AM) and plated on different matrix ligands. Emitted fluorescence was measured over time (fig. 1). Each matrix tested caused the release of a unique concentration of cytosolic calcium, and all were higher than cells plated on poly-L lysine. This is presumably due to the subtypes and quantities of integrins engaged. In order to demonstrate that simple clustering of integrin subtypes is also capable of stimulating variable cytosolic Ca^{2+} flux, cells pre-incubated with Fluo-3 AM were plated in wells coated with antibodies directed against known laminin-1 binding integrins and intact laminin-1. Each of the integrins that influenced adhesion to laminin-1 stimulated an intracellular calcium release that was different from binding of the intact molecule (fig. 2). These results suggest that signaling events downstream of integrin activation synergistically determine the ultimate concentration of cytosolic calcium release upon matrix engagement.

Fluo-3 AM is a cell permeant dye whose excitation is increased upon binding free calcium. The acetomethyl (AM) ester is cleaved in the cytosol, thus restricting the dye from entering intracellular calcium stores. While intracellular calcium release occurs with release of distinct concentrations and with frequencies of distinct period [19], this dye is capable of measuring only total release of calcium over time under the conditions used [20]. With a high affinity for calcium, (Kd=325nM), this dye is likely to bind all free cytosolic calcium [20]. Fluorescence measurements therefore reflect accumulation of total calcium released by flux of calcium release over time. The fluorescence measurements reported are not likely to have arisen from changes in pH. The test media was buffered (see materials and methods), Fluo-3 AM is non fluorescent in the absence of calcium and is relatively insensitive to changes in pH. In fact, this dye is used with pH sensitive dyes for simultaneous calcium and pH determinations [20, 21].

At the end of each Ca^{2+} determination assay, cells were washed vigorously in PBS and the number of cells remaining were counted as in adhesion assays (data not shown). The number of adherent cells was roughly inversely proportional to cytosolic

calcium release. Laminin-5 is the preferred adhesive substrate of these cells [22], and caused the least release of cytosolic calcium of all the matrices tested (Fig. 1). This suggests that cells loosely adherent or actively migrating within the well released more frequent calcium fluxes, or more calcium per release event.

MCF-10A engagement to laminin-1.

To determine the binding properties of MCF-10A cells on laminin-1, cells were pre-incubated with function blocking antibodies directed against integrins expressed by MCF-10A cells, a chelator of cytosolic calcium (BAPTA-AM), and the PI-3 kinase inhibitor wortmannin (Fig. 3). Cells were also incubated in heparin sulfate to demonstrate that adhesion to laminin-1 is not dependent upon non-specific charged proteoglycan-heparan interactions. Blocking integrins □1, □6, and □1 inhibit adhesion of MCF-10A cells to laminin-1 indicating that these are the primary integrins that determine the strength of adhesion to laminin-1. □6, which can form a heterodimer with □4, are binding laminin-1 in the □6□1 configuration, as blocking □4 has no effect on adhesion (Fig 3. Lane 14). Although not statistically significant, blocking □3 appears to increase the strength of MCF-10A cell adhesion to laminin-1. Integrin □3 is included in these studies as a potential laminin-1 binding molecule due to reports that □3 acts as a negative regulator of □6 activity in other cell lines [3]. MCF-10A engagement to laminin-1 is dependent upon cytosolic calcium as pre-incubation with BAPTA-AM abolishes adhesion (Fig. 3, lane 3). Integrin mediated release of cytosolic Ca^{2+} is partially dependent upon the activity of PI-3 Kinase as wortmannin, a specific inhibitor of PI-3 kinase at the concentrations used, blocks adhesion in a concentration dependent manner (Fig. 3, lanes 4,5).

Laminin-1 domains as putative integrin engagement sites.

A comprehensive analysis of structure/function studies conducted with EHS laminin-1 indicates that cellular function can be localized to distinct globular domains on each arm of the laminin-1 subunits (Fig. 4) [23-50]. These studies were conducted with either protease cleavage fragments of laminin-1 or small overlapping peptides derived from the laminin-1 sequence. Only rarely was a specific fragment or peptide implicated as a ligand of a specific integrin subtype. Because the large protease cleavage fragments are likely to be bound by more than one integrin subtype, and because small peptides are unlikely to form conformations similar to the native molecule, I decided to produce several individual domains in a eukaryotic expression system. The baculovirus expression system was chosen for ease of use, protein yield, and because proteins expressed in this system are generally processed in a manner similar to vertebrate cells. Domains □VI (AVI), □IVb (AIVb), G1, G3, G4, and G5 were chosen due to the high probability of having biological activity.

Production of recombinant laminin-1 domains.

Six proteins derived from the EHS laminin-1 sequence corresponding to specific globular domains were produced. Based on silver-staining of protein elutions, each protein was purified to greater then 95% purity (data not shown). Protein yields varied from 2-10□g/L. The molecular weight of each protein conforms closely to the predicted size following cleavage of carbohydrate chains (fig. 5). All six proteins are glycosylated via N- and not O-linkages based on gel shifts following cleavage by pan-specific N- and O- glycosidases.

Recombinant domains of laminin-1 produced by baculovirus expression do not support integrin mediated adhesion of MCF-10A and MDA-MB-231 cells.

In order to screen each recombinant protein as an adhesive substrate for epithelial cells, MCF-10A cells were plated in wells coated with purified proteins and intact laminin-1. Due to low concentration of recombinant proteins, wells were coated with equimolar amounts of protein (21.1nM), and not identical masses. Statistically reliable adhesion to recombinant proteins by MCF-10A cells was not demonstrated (Fig. 6). Manganese, which acts to strengthen integrin engagement, did not influence MCF-10A adhesion to the recombinant proteins (data not shown). When pre-incubated with MCF-10A cells, no recombinant protein influenced adhesion to laminin-1 (data not shown). In order to determine if the failure to bind the recombinant domains was cell line-specific, MDA-MB-231 cells, which bind to laminin-1 more strongly than MCF-10A cells, were also tested for adhesion. As indicated by pre-incubation with function blocking anti-integrin antibodies, MDA-MB-231 cells engaged laminin-1 with the □6□1 integrin (fig. 7, lanes 2-8). These cells did not, however, bind to proteins A6-G4 to any greater extent than negative control (fig. 7 lanes 9-13). These cells did show significant adhesion to protein G5. This binding, however, was integrin-independent as function blocking anti-integrin antibodies failed to influence adhesion (fig 7. Lanes 14-19). Curiously, MDA-MB-231 adhesion to G5 was also independent of proteoglycan-heparan charged interactions (fig 7, lane 20). This indicates that these cells engage protein G5 via a member of another family of adhesion receptors, such as E-cadherin, □-dystroglycan, or CD44.

Because epithelial cell adhesion to substrate normally results in formation of focal complexes and spreading, MDA-MB-231 cells were incubated on laminin-1 and protein G5 for 90 minutes, and visualized by fluorescence microscopy. Focal complex formation was visualized indirectly with anti-vinculin antibody, and actin structure was visualized by staining the actin cytoskeleton with fluorescein-conjugated phalloidin (fig. 8). MDA-MB-231 cells spread and formed distinct focal complexes on laminin-1 (fig. 8, panel A,B). On protein G5, adherent cells remained rounded and did not form any distinct cytoskeletal structures or focal complexes (fig. 8, panels C,D).

Discussion

Failure of the recombinant proteins to support adhesion may be due to altered conformations, low concentration, or artifacts of the baculovirus expression system. Because sf9 are invertebrate cells, it is possible that the proteins are processed differently than they would be by vertebrate cells. The baculovirus system, however, uses many of the protein modification, processing, and transport systems present in higher eukaryotes, including myristilation, palmitilation, phosphorylation, protein targeting, and cleavage of signal sequences. A difference of concern, however, is that N-linked oligosaccharides in insect cells are only high mannose type. They are never processed to the complex oligosaccharides containing fucose, galactose, and sialic acid seen in vertebrate cells. This is only of concern if integrins require the presence of specific carbohydrate structures for ligand recognition. Non-glycosylated laminins have been reported to be adhesive [51], and non-adhesive (Y. Yamada, personal communication). O-linked glycosylations have been shown to occur in insect cells, but are less well characterized [52, 53].

In addition to altered glycosylation, the low yield of my system could also be responsible for the lack of biological activity of these proteins. In the construction of

all recombinant viruses I chose the late gp64 promoter, as opposed to the more common very late polyhedrin (polH) promoter. The gp64 promoter typically produces 60% the yield of the stronger polH promoter but is active at a stage of viral infection when the protein processing machinery required for efficient production of secreted proteins remains intact [54]. The small size of the proteins (16-45kD) also contributed to low yield when measured by weight, as smaller sequences are transcribed no more frequently than longer sequences preceded by the same promoter region. Small proteins stored at low concentrations also tend to be less stable over time and less able to withstand freeze-thaw cycles. The low yield necessitated the use of low concentrations for all assays. All intact laminin-1 was used at a concentration of 20□g/ml, while each domain protein was used at ~1□g/ml, which represents a 1:1 domain/intact laminin-1 equimolar ratio (21.1nM). MCF-10A and MDA-MB-231 cells, however, both adhered to laminin-1 plated at 21.1nM to nearly the same extent as when plated in wells coated with 20□g/ml laminin-1 (data not shown).

Although yield with the baculovirus system is unpredictable and varies greatly among different proteins expressed, the yield in our system could be improved by double infection of sf9 cells with viruses containing the domain sequence downstream of both the polH and gp64 promoters. This strategy would extend the time of protein expression during viral infection and take advantage of the stronger polH promoter, but would require constructing, purifying, and amplifying an additional six viruses. A prudent continuation of this work would be to produce proteins from the same DNA sequences described here in another system that may provide a greater yield. Other protein expression systems that may prove successful include direct transfection of bacterial, *Drosophila melanogaster*, yeast, or mammalian cells.

The adhesion of MDA-MB-231 cells to protein G5 may be due to specific laminin-dystroglycan interaction. Dystroglycan is a heterodimeric integral membrane glycoprotein receptor for several ECM molecules including laminins. It is expressed in many epithelial cell types and binds laminin through G domains. In mice knockout studies, it is shown that laminin-1, □1 integrins, and dystroglycan are required at the same development stage, when the basement membrane first appears [55]. In addition, it is demonstrated that dystroglycans have affinity for laminin in the low nanomolar range [56].

The use of synthetic peptides is omitted here as a realistic model of integrin ligands. Although they are widely demonstrated to effectively inhibit tumor cell adhesion to ECM and other cells, stimulate anoikis, and decrease tumor cell metastases *in vivo*, they are not demonstrated to be activators of integrin function. For example, the binding of a peptide that blocks adhesion to laminin is itself blocked by only the □6 and not the □1 anti-integrin antibodies [23]. In addition, most integrins require either magnesium or calcium in sites away from the binding domain. For this reason, integrin mediated adhesion is typically blocked by EDTA. Adhesion to integrin binding synthetic peptides are not [23]. This data indicates that the peptides are functioning to block the integrin binding sites but are not mimicking the binding to an intact native ligand. Lastly, the minimum size of fibronectin derived peptide that still preserved the total binding activity is a fragment with a molecular weight of 75kDa [57].

While the recombinant proteins generated proved not to be reliable integrin substrates, I believe that the generation of recombinant proteins that can activate specific integrin subtypes by natural receptor/ligand interaction on a variety of cell types will be valuable integrin research tools. I favor this strategy over the production of transfected cells that express only defined integrin subtypes as recombinant cells

expressing foreign integrins may not have the cellular machinery required to propagate native integrin-stimulated signals. In addition, recombinant domains would be more flexible research tools as they could be used with several cell types as well as in different combinations, without having to produce doubly and triply transfected cell lines. Lastly, recombinant domains could be useful for more than just the study of adhesion, but other functions relevant to cancer progression including cell cycle control, prevention of apoptosis and migration-associated tissue remodeling [58].

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Appendix 3

Title: The promise of integrins as effective targets for anti-cancer agents

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Running Title: Integrin receptors: unrealized chemotherapeutic targets

Abstract

This review will briefly describe integrin function, address why integrins are attractive targets for chemotherapeutic drug design, and discuss some ongoing studies aimed at inhibiting integrin activity. Integrins are cell surface heterodimeric receptors. They modulate many cellular processes including: growth, death (apoptosis), adhesion, migration, and invasion by activating several signaling pathways. Many potential chemotherapeutic agents target integrins directly (e.g., polypeptides, monoclonal antibodies, adenovirus vectors). These agents may be clinically useful in controlling the metastatic spread of cancer.

The primary therapy for most solid tumors is surgical resection, followed by a combination of radiation and chemotherapy. Most of the currently used chemotherapeutic agents target rapidly dividing cells. However, many solid tumors are not rapidly dividing and thus evade these agents. Often, tumors that resist or evade chemotherapy treatments continue to grow, and may spread to other, distant organs, resulting in the formation of secondary tumors (metastases). Once a cancer develops to this stage, patient prognosis is usually very poor. Novel chemotherapeutic agents need to be developed to help control the growth and spread of metastatic tumors. In this review, we discuss the potential value of integrins as chemotherapeutic targets. The function of integrins in cancer progression is addressed, followed by a discussion of current drug discovery efforts and clinical trials of compounds that specifically target integrin receptors.

The latest generation of chemotherapeutic agents is designed to target molecules required for survival by cancerous cells but not by normal cells. One very interesting group of molecules that are receiving attention is the integrin family of cell surface adhesion receptors. In the past 10 years, it has become clear that the integrins play an important role in virtually every stage of cancer progression. In addition, oncogenic transformation is often accompanied by changes in integrin expression and substrate preference. Although integrins are not oncoproteins, they modulate the processes of cell growth, death, migration, and invasion, which all impinge on the severity of clinical disease [1]. Several drugs in clinical trials function as integrin antagonists; all having shown promise as anti-angiogenic, anti-metastatic, and anti-proliferative compounds in mouse models [2].

Integrin structure and function

Integrins are a family of heterodimeric cell surface receptors. The known 18 □ and 8 □ subunits combine to form at least 24 □□ heterodimers (for recent reviews see references 2-4). Most cells express more than one type of integrin heterodimer. Integrin expression profiles are unique for distinct cell types, and change with developmental stage and physiological conditions within a cell type [5]. Most integrins mediate cell/substratum adhesion by binding to extracellular matrix (ECM) proteins, while a few mediate cell-cell adhesion via homotypic or heterotypic coupling with other cell surface receptors. This family of receptors can be classified into three subfamilies. The □1 integrins generally mediate interactions between cells and ECM. The □2 integrins are restricted to leukocytes and are typified by having other cell surface proteins as their ligands. The □3 integrins are almost exclusively expressed on platelets and megakaryocytes and act as important mediators of platelet adhesion [6]. Excepting the fibronectin receptor □5□1, all integrins bind to more than one ligand. Each ECM molecule is also bound by more than one integrin. Although it is impossible to predict an integrin binding site based on sequence, an acidic residue is common to all known binding sites, and many contain the sequence RGD [2].

At sites of integrin activation and clustering, protein aggregates termed focal complexes and focal adhesions assemble on the intracellular surface. The types of proteins that form these complexes can be grouped as either structural, which form links to the actin cytoskeleton, or signaling, which include a variety of kinases and adapter molecules linking integrins to other kinases, members of the GTPase families, lipid kinases and phospolipases, and ion channels [7,8]. The structural components are talin, □-actinin, vinculin, and filamin. Of these, only □-actinin binds directly to integrins, while the others are capable of binding each other and members of the

signaling component [9,10]. These complexes are sites where the cell can generate tension with respect to its surroundings, allowing the cell to alter its shape and carry out complex processes such as migration and cell division [2].

Integrin clusters are signaling complexes

The signaling proteins found at focal complexes and focal adhesions associate with integrin cytoplasmic domains to form a nexus for stimulating intracellular signaling cascades. At these sites, signaling from outside to inside the cell occurs which contributes to the regulation of diverse cellular processes including entry into the cell cycle, programmed cell death (apoptosis), gene transcription, regulation of intracellular pH, differentiation, and migration. A summary of the known signaling pathways associated with integrin receptors is presented in Figure 1. Signaling through integrins from the inside to the outside of the cell regulates adhesive strength through affinity and avidity modulation, and helps in remodeling of the ECM during tissue development and tissue invasion. Exactly how this occurs is unknown, but phosphorylation of integrin subunits may be important in both processes [6].

The 125kDa kinase termed focal adhesion kinase (FAK) is the best characterized of the integrin-associated signaling proteins. FAK binds to integrin receptors and plays a central role in assembling complexes of signaling proteins at the cell surface. FAK has numerous tyrosine residues that, when phosphorylated, act as docking sites for the recruitment and activation of several SH2- and SH3-containing classes of signaling molecules. Talin and paxillin are implicated in the activation of FAK by stimulating its autophosphorylation on Y397. This opens a binding site for the oncoprotein Src. All three of these molecules are implicated in targeting FAK to the focal adhesion. Src also phosphorylates FAK on a number of residues, including Y925. The adaptor protein Grb2 binds to phosphorylated Y397, and through the action of the guanine nucleotide exchange factor SOS, connects activated receptor tyrosine kinases to Ras/ERK/MAP kinase cascades, [2,4,8,11]. Phosphatidyl Inositol 3-Kinase (PI-3K) also binds to Y397, and mediates cell proliferation, cell migration, and apoptosis through its downstream effector PKB/Akt [3,5]. The adaptor protein $p130^{CAS}$ is a substrate of the FAK/Src complex and is implicated in activating the ERK cascade as well as Jun N-terminal kinase (JNK). Both $p130^{CAS}$ and FAK are thought to use the ERK pathway to activate several transcription factors [4]. FAK is also implicated in controlling cell cycle progression and preventing apoptosis through a pathway involving protein kinase C, phospholipase A2, and p53 [4, 12].

Integrin regulated signaling proteins that can be activated independent of FAK include protein kinase C, integrin linked kinase, integrin associated protein, and the tetraspan (TMS4F) adapter proteins [2,11]. Integrin linked kinase appears to phosphorylate only integrin subunits, and may function as a bridge to FAK [2]. Although tetraspans can regulate cell motility, their mechanism of action is not understood.

Integrins contribute to cancer progression

Integrins act to promote the growth, and retard the death, of both normal and tumorigenic cells. In cooperation with growth factor receptors, they induce proliferation by specifically causing the transcription of the cyclins and cyclin dependent kinases (Cdks) required for transition past the G1 checkpoint, and by down-regulation of Cdk inhibitors [2,6,12]. The fact that most cells deprived of ECM interactions undergo apoptosis (programmed cell death), and that integrin ligation can rescue those cells, demonstrates that integrins are involved in stimulating apoptosis

resistance mechanisms [2]. For instance, in some cell lines, integrins activate transcription of the Bcl-2 gene and increase the activity of MAPK, JNK, PI-3 kinase, and PKB/Akt, all of which are known to inhibit apoptosis [2,3,5,6]. Lastly, cell death by deprivation of engagement of ECM (anoikis) may be an important control mechanism in cancer since carcinoma cells that lose contact with the matrix would die rather than circulate and colonize distant sites [3,5]. Hence, changes in the integrin expression profile may dramatically influence the progression of malignant tumors.

Benign tumors are encapsulated by an organized lattice of basement membrane components. The progression to malignancy, and the clinical diagnosis of malignant disease, is essentially determined by the capability of tumor cells to dissociate, degrade the lattice, and metastasize to other locations within the body. This process, termed the metastatic cascade, begins with the detachment of single tumor cells and active infiltration by those cells into the surrounding stroma where entry into the vasculature and lymphatic system is possible. Dissociation of individual cells from a tumor mass is regulated by the E-cadherin family of receptors. These are shown to be suppressors of epithelial tumor metastasis. Recently, activation of integrin □3□1 was shown to down-regulate E-cadherin mediated adhesion, causing loss of cell-cell adhesion, junctional communication, and enhancing invasiveness of malignant tumor cells [13]. For invasion by dissociated cells to occur, the ECM that surrounds the neoplastic tissue must be degraded to allow the escape of invasive cells. Integrins participate in the ECM degradation by stimulating the secretion of ECM-degrading proteases such as matrix metalloproteases, and enhancing invasion through a signaling cascade involving Ras [2,3].

Integrins as therapeutic markers

The large number of investigations into the effectiveness of integrins as indicators of disease and as an aid to non-invasive cancer imaging underlines the potential usefulness of these receptors in the clinic. Several recent studies show that both upregulation and downregulation of integrin expression can be effective markers of incidence of disease and patient prognosis. A comparison of normal and neoplastic human prostate tissues showed a downregulation of a specific variant of the □1 integrin subunit [14], and strong evidence shows that reduced expression of □6 and □4 may contribute to the higher tumorigenicity of androgen independent prostate tumor cells [15]. In a study of metastatic melanoma, longer disease free survival and overall survival correlated with □1 expression [16], while neuroblastoma aggressiveness was correlated with expression of integrin □v□3 and □v□5 by the microvascular endothelium [17]. Studies of acute lymphoblastic leukemia show that □2 expression was significantly associated with splenomegaly [18], and expression of □5□1 was associated with positive response to chemotherapy in patients with rectal cancer [19]. Finally, node negative non-small cell lung cancer patients whose tumors over-express integrin □5 had a lower survival rate than those whose did not [20].

These clinical correlations of patient biopsies to integrin expression are recapitulated to some degree in rodent and in vitro analyses. For example, a study of rat hepatocarcinogenesis showed that integrins □1 and □5 were upregulated in metastases of the lung and diaphragm, while integrins □1, □2, □3, and □5 were decreased in the primary carcinoma and preneoplastic nodules [21]. Among pancreatic cancer cell lines, those that showed a higher potential to metastasize in a mouse model had enhanced expression of □v□5 while those that did not metastasize had enhanced expression of □3 [22]. The expression of □4 was inversely correlated with dissemination of ten human gastric cancer cells lines in SCID mice [23], and two

of three human epithelial ovarian cancer cell lines could be identified by high expression of □v□6 integrin, which was correlated with integrin linked signaling and protease secretion [24]. The development of endometrial cancers has been linked to the loss of progesterone receptors. It was found that re-expressing an isoform of that receptor inhibited the expression of integrin □3, □1, and □3 subunits and concomitant inhibition of cell invasion into matrigel [25].

In addition to the cases mentioned above, integrin expression can also be specific to levels of tumor resistance to common anti-cancer chemotherapies. Gastrointestinal tumor cells selected for resistance to 5-fluorouracil showed marked under-expression of the integrin □3 [26] while human ovarian cancer cell lines resistant to the fenretinide were associated with reduced expression of □1 integrin [27]. A nasal carcinoma cell line resistant to melphalan showed increased expression of □2, □5, □6, and □4, decreased expression of □4 and exhibited enhanced invasion in vitro [28]. Glioma cell lines resistant to vincristine, doxorubicin, and etoposide, showed enhanced expression of integrins □2, □3, □5, and □1 [29].

If the integrin receptors expressed by a particular tumor are reproducibly correlated with patient prognosis, that knowledge can be used by the clinician to choose the appropriate therapeutic regimen. Non invasive imaging based on integrin expression is potentially useful not only to identify particular types of tumors but to assess their responsiveness to particular drugs or drug-targeting methods. In two recent studies, a glycopeptide containing the integrin binding domain RGD was found in tumor mouse models to be suitable for tumor visualization and determination of integrin status [30], and a similar peptide specifically bound to □v□3 expressing tumors in vivo [31].

Integrins as targets for drug delivery and gene therapy

Achieving tissue-specific drug delivery is the primary challenge faced by researchers in the fields of gene therapy, targeted drug delivery, and immune meditated tumor destruction. Integrin expression can both provide solutions to and exacerbate the difficulty of this problem. For example, the targeting and activation of lymphoid cells to tumor sites is dependent upon binding through integrin receptors, which in turn can be modulated by secreted chemokines [32]. In a unique mixture of strategies, infection of mice with adenovirus carrying IL-12 gene and targeted to □v□3 integrin expressed on liver metastases led to enhanced recruitment of adoptively transferred cytolytic T lymphocytes [33]. In another case, a human transitional cell carcinoma cell line transfected to over-express IL-6 showed concomitant increases in □5 and □1 expression, which increased the adherence of tumor destroying cells of bacillus Calmette-Guerin therapy [34]. A chimeric cell adhesion molecule containing the □v□3 disintegrin kistrin and CD31/PECAM-1 served as a bridge to home adoptively transferred lymphoid cells to angiogenic endothelial cells and caused the accumulation of lymphoid cells to angiogenic tumors in lewis lung and melanoma mice models [35].

Another excellent example is the use of recombinant adenoviruses that interact with cell surface integrins. Some adenoviruses are successfully internalized into host cells by forming complexes with the host proteins CAR (Coxsackie B virus and Ad Receptor) and integrins □v□3 and □v□5 [36]. The semi-specific expression of these proteins makes targeting difficult. In some cases replacing the binding capacities of the viral capsid may prove effective while in others it may be effective to enhance the integrin binding capacities. Using the former strategy, an adenoviral vector targeted solely to the human epidermal growth factor receptor showed improved targeting to

tumors of the brain [37]. But an adenovirus vector targeted to CAR, epidermal growth factor receptor, *and* □v integrins via the RGD peptide showed enhanced gene transfer efficiency to pancreatic carcinoma and glioma cells [38, 39]. In a study of adenoviral gene transfer efficiency in ovarian cancer cells, poorly infected cell lines showed no expression of □v□3 integrins, and re-expression of these receptors increased adenoviral infection [40]. In order to target cells not expressing CAR or □v integrins, an antibody specific for the adenoviral vector was fused with recombinant growth factors to serve as a bridge for targeted infection [41]. Recently, integrin receptors expressed in large numbers on most ovarian cancers (□v□3 and □v□5), where used as binding targets for adenovirus vectors containing the herpes simplex virus thymidine kinase gene for molecular chemotherapy [42]. These vectors were very effective against purified primary ovarian cancer cells and may be a useful agent for treating ovarian cancer in clinical trials.

Finally, in an effort to target a recombinant virus that does not use integrins in the process of internalization, a capsid of the feline panleukopenia virus was modified to express the RGD motif to bind □v integrins. This successfully contributed to directing infection of those particles to a human rhabdomyosarcoma cell line [43].

Non-viral strategies for disrupting integrins

Non-viral and non-lymphoid strategies for integrin-specific tumor targeting focus on integrin-specific antibodies, soluble integrin ligands, or vectors that encode cyclic glycopeptides that contain the integrin binding RGD sequence. A vector with the RGD motif was found to preferentially colocalize to tumor tissues over the lung, kidney and spleen in nude mice (18). In lysosomal vectors that resemble retroviral envelopes but are not-toxic, this motif was shown to aid transduction of human melanoma cells (19). The anti-microbial peptide tachyplesin coupled to this motif was effective in inhibiting the growth of tumors in a mouse model and inducing apoptosis of prostate cancer cells in vitro (34). Alone, an RGD containing peptide caused anoikis of glioblastoma cell lines and prolonged the survival of intracranial tumors in SCID mice (27). And finally, RDG containing peptides have shown promise as drug delivery agents for radiotherapy. This is thought to be effective because irradiated tumor blood vessels are found to have activated □IIb□3 integrins. At least one of these peptides, biapcitide, is currently in clinical trials (36).

Disintegrin is the name given to soluble integrin ligands (originally isolated from snake venom) that disrupt cell-matrix interactions [28]. One disintegrin, contortrostatin, disrupts cytoskeletal structure and hence inhibits cell motility, raising the possibility that these compounds may be useful for therapeutic intervention for cancer invasion and metastasis [29]. Abciximab is a mouse-human chimeric monoclonal Fab fragment approved for adjunct therapy for the prevention of cardiac coronary intervention. Abciximab binds to the integrin □$_{IIb}$□□GPIIbIIIa) receptor on platelets which is the major adhesion receptor involved in aggregation. It also binds two other integrin receptors: the □v□3 receptor (present in high density on activated endothelial and smooth muscle cells) and □M□2 integrin (present on activated leukocytes) [30]. It is reasonable to assume that Abciximab may have anti-metastatic properties in cases of tumors that express the above integrin receptors.

The specificity of these inhibitors varies for different types of integrins. Therefore, to develop and then effectively utilize an anti-integrin therapy, the type of integrin and/or effectiveness of each agent or combination of agents must be measured. We have developed a fluorescence-based automated assay for identifying antimigratory compounds with the ability to discern cytotoxic from noncytotoxic

modes of action that can achieve this goal [32]. The assay utilizes a chambered well that can be used to simultaneously measure migration and viability of cells following treatment with inhibitors. This enables us to assay the effects of compounds that disrupt integrins directly, or that interfere with downstream signaling events following integrin ligation. For example, with this assay we have been able to show that carboxyamidotriazole, a calcium channel blocker, inhibits chemotactic and haptotactic migration of breast cancer cells more effectively than tamoxifen (an antiestrogen). This assay should increase the ability to rapidly screen chemical libraries for even more compounds that inhibit integrin function.

These are but a few examples of agents currently in development to target integrins as anticancer therapies. These agents hold the promise of being effective, selective, and highly tolerable in adjuvant therapies.

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